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Review

Reversed-phase high-performance liquid chromatography of purine compounds for investigation of biomedical problems: application to different tissues and body fluids

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ABSTRACT

An overview of high-performance liquid chromatographic separation techniques (reversed-phase and ion-pair reversed-phase) used in the analysis of purine ribonucleotides, ribonucleosides and nucleobases, including procedures for sample preparation, is given. Coverage of the separation techniques is extended to the measurement of specific radioactivities of these compounds in tracer kinetic experiments for metabolic flux rate analyses. This article is focused on the development and adaptation of reversed-phase separation techniques for nucleotides, nucleosides and bases that are used to examine a variety of biomedical problems. The investigation of purine nucleotide metabolic disorders or physiological transitions in the pathomechanisms of different diseases and syndromes or in cell maturation processes, respectively, requires the application of chromatographic separation to a multitude of tissues and body fluids. These samples vary greatly in concentrations of purine compounds with low molecular mass, from *ca.* 5 m*M* to *ca.* 0.5 μM. The advantages and disadvantages of different techniques are critically discussed.

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LIST OF ABBREVIATIONS

Ade	Adenine
Ado	Adenosine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMP-Succ	Adenylosuccinate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CE	Capillary electrophoresis
DNA	Desoxyribonucleic acid
GC	Gas chromatography
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
Gua	Guanine
Guo	Guanosine
HPLC	High-performance liquid chromatography
Hyp	Hypoxanthine
IMP	Inosine monophosphate
Ino	Inosine
NAD^+	Oxidized nicotinic acid amide adenine dinucleotide
NADH	Reduced nicotinic acid amide adenine dinucleotide
$NADP^+$	Oxidized nicotinic acid amide adenine dinucleotide phosphate
NADPH	Reduced nicotinic acid amide adenine dinucleotide phosphate
N.D.	Not determined
^{31}P NMR	^{31}P nuclear magnetic resonance spectroscopy
RCM	Radial compression module
rhEPO	Recombinant human erythropoietin

RNA	Ribonucleic acid
TLC	Thin-layer chromatography
TNB	Trinitrobenzenesulphonic acid
UA	Uric acid
UV	Ultraviolet
Xan	Xanthine
Xao	Xanthosine

1. INTRODUCTION

Intact cells contain *ca.* fifty low-molecular-mass purine compounds with a concentration range from 5 m*M* (ATP in some tissues) to less than 1 μM (adenosine). The common purines and their metabolic pathways are shown in Fig. 1. The scheme includes the metabolic pathways of adenine and guanine ribunucleotides and their degradation products. The transport mechanisms of purine nucleosides and bases, and the pathways of purine ribonucleotide metabolism, have been described extensively in various books and review articles [1–8].

There are several diseases that are due to or connected with changes in purine ribonucleotide metabolism. One of the first known diseases of purine metabolism was gout [9]. Today it is known that gout is a disease of civilization, resulting from a bountiful supply of purines. The concentration of uric acid in blood plasma and tissues is dependent on the purine content of food; to a great extent, on its DNA and RNA content. The different forms and symptomes connected with gout are discussed in detail in ref. 10.

Other diseases connected with disturbances in the purine pattern of cells and/or blood plasma involve enzyme deficiencies in purine metabo-

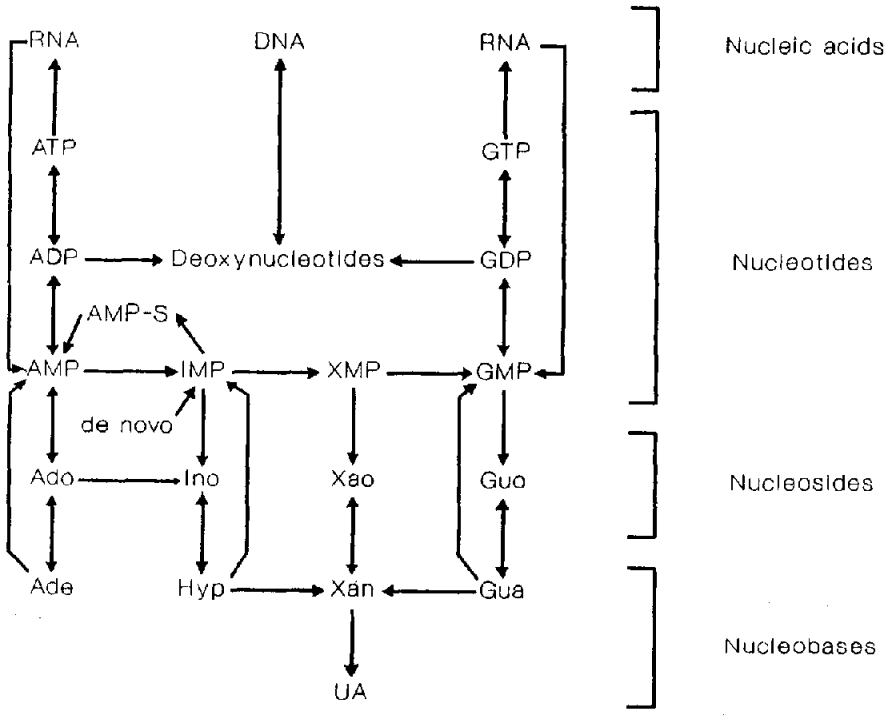

Fig. 1. Pathways of purine metabolism in human cells.

TABLE 1

SOME DISEASES AND SYNDROMES ASSOCIATED WITH CHANGES IN PURINE METABOLISM

Disease (syndrome)	Reference(s)
Gout	9,10,20
Xanthineuria	10
Enzyme deficiencies	11,12,21–25
Hypoxanthine guanine phosphoribosyltransferase	
AMP-deaminase	
Adenine phosphoribosyltransferase	
Adenosine deaminase	
Purine nucleoside phosphorylase	
Oxygen deficiency	13–16,26,27
Systemic hypoxia (*e.g.* perinatal asphyxia)	
Local hypoxia (ischemia of organs)	
Degradation of microcirculatory disturbances (in many diseases)	
Intoxication or cytotoxic side effects of drugs, *e.g.* phenylhydrazine, haloalkanes, cyclosporine A, uremic toxins	28,29
Inflammation (e.g. inflammatory bowel disease) and oxidative damage of DNA (8-OH-guanine formation)	30–33
Oncological diseases	17–19,34–37
Tumour proliferation	
Tumour markers	
Antitumour therapy (antimetabolites)	

lism. The known forms of such genetic enzyme-coding errors are shown in Table 1. Most changes in purine-metabolizing enzymes are connected with diseases, such as muscular distrophy (AMP-deaminase deficiency) and immunological disorders (adenosine deaminase or purine nucleoside phosphorylase deficiency) [11,12].

Furthermore, some diseases and symptoms (Table 1) are connected with changes in purine nucleotide metabolism due to ATP depletion during oxygen deficiency (ischemia or hypoxia) [13–16]. Markedly increased nucleotide turnover during proliferating phases of tumour growth [17–19] results in enormous changes of purine nucleotide, nucleotide and base patterns both in the tumour and in the host tissues.

2. METHODS FOR THE ANALYSIS OF PURINE NUCLEOTIDES

To find an optimal procedure to analyse purine compounds one has to solve a group of problems concerning (i) the multitude of purine compounds present in the analysed tissue in detectable amounts, (ii) the great concentration differences, (iii) the differences of distribution of compounds inside/outside and in the different compartments of the cells, (iv) the high turnover of purine metabolic pathways (*e.g.* ATPases) and (v) the different enzymes of different cell types and tissues.

The commonst procedures for analysis of nucleotides and their degradation products are listed in Table 2. In medical practice and biomedical research, enzymic spectrophotometric or fluorimetric measurements and determination of purine compounds by means of high-performance liquid chromatographic (HPLC) separation techniques have been applied most frequently [38–42]. For specialized diagnosis of heart, liver and other organs, ^{31}P NMR spectroscopy has been used [16]. The most significant advantages of purine analysis by HPLC are the separation of a great variety of metabolites and a detection sensitivity that allows the analysis of very small samples. The equipment is easy to handle and makes possible the rapid and complex analysis of purines in a variety of biological samples. HPLC also allows the study of the dynamics of purine metabolism within short time intervals.

TABLE 2

METHODS OF PURINE DETERMINATION

Method	Compound	Remarks
Enzymic determination	*e.g.* ATP	In most cases only one compound
	Uric acid	High specificity and sensitivity
		Different detection modes (spectrophotometric, bioluminescence, fluorimetric)
^{31}P NMR	Nucleotides	Without cellular destruction (non-invasive method)
		Metabolite groups
TLC	Nucleotides	Low resolution
HPLC	All compounds	Wide spectrum of metabolites
GC	Nucleosides	High sensitivity
		Only selected metabolites
CE	All compounds	Microanalysis
		Low sensitivity

The disadvantage of all the methods mentioned above in comparison with the non-invasive nuclear magnetic resonance spectroscopy (NMR) technique is the necessity to remove samples of cells, tissues or body fluids. One of the modern invasive microanalytical approaches to determine also purine compounds is capillary electrophoresis [43]. The most frequently used detection mode in HPLC analysis of low-molecular-mass purine compounds is UV detection. An absorption maximum of adenine- and guanine-containing compounds at 254 nm has often been used for analyses. The absorption maximum of oxypurines (hypoxanthine, xanthine and uric acid) is at a higher wavelength, 292 nm, which is useful for the detection of uric acid. In addition to detection at 254 nm, a parallel detection wavelength of 280 nm has been used. The absorption ratio A_{254}/A_{280} is specific for certain purine compounds: for adenylate compounds this ratio is *ca.* 4.1, for guanylate compounds it is *ca.* 1.8 [44]. Several authors have used fluorescence detection [45,46] which is highly selective and very sensitive. Electrochemical detection can be very powerful, particularly for bases [47–49].

Anion-exchange and reversed-phase HPLC are the principal methods used for nucleotide separation. Both have their advantages and disadvantages. The following discussion will focus on methodological applications of reversed-phase HPLC in the analysis of various biological samples.

3. REVERSED-PHASE HPLC SEPARATION TECHNIQUES FOR PURINES

3.1. Sample preparation

The sample preparation involves, in general, a fast stop of enzymic reactions and extraction of proteins to provide a clean supernatant for HPLC injection. The method mostly used for deproteinization is extraction with perchloric or trichloroacetic acid [44,50,51], but it has the disadvantage that it destabilizes reduced pyridine nucleotides. If one needs to analyse the reduced forms of pyridine nucleotides the method of choice for deproteinization is extraction by KOH [51]. Both methods are outlined in Fig. 2. Extraction with organic compounds has also been used, but it is not as effective for the removal of proteins as either of the other two procedures: the supernatant obtained is not always clear [52]. For estimation of recovery of extracted procedure several internal standards have been used [53]. It is also possible to analyse small volumes of body fluids without any extraction. Direct injection of body fluid samples has been described, especially for urine and cerebrospinal fluid, and also for plasma [54]. Here, large proteins could be removed by a filtration step [55].

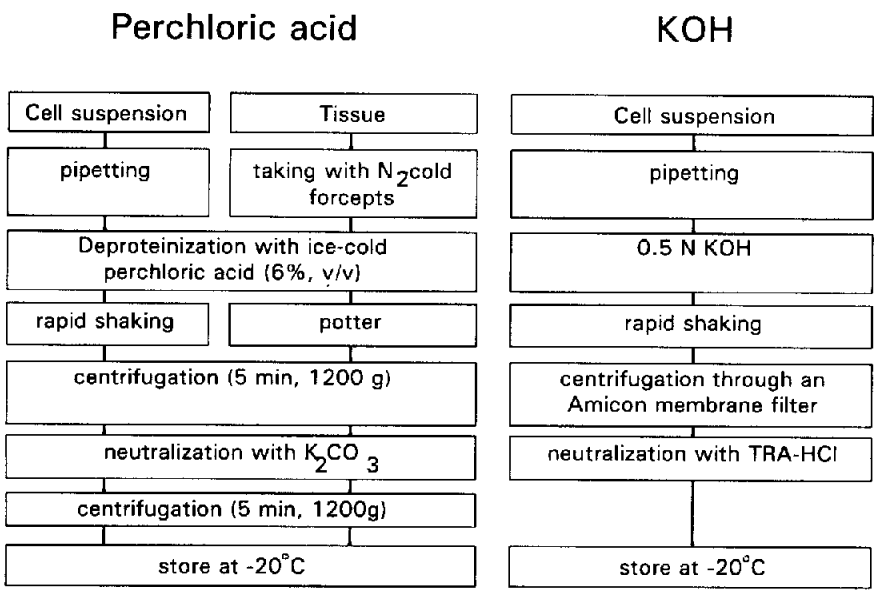

Fig. 2. Scheme of extraction procedures with perchloric acid and potassium hydroxide. (From ref. 51.)

If one compares the effectiveness and simplicity of the extraction procedures, the method of choice in the most cases is the acidic extraction, which is performed with trichloroacetic or perchloric acid. In the case of trichloroacetic acid, the supernatant can be neutralized with freon or serial extraction with diethyl ether. Using perchloric acid, the perchlorate ions can be removed by addition of potassium ions (KOH or K_2CO_3). But the analyst must complete a methodological scheme (Fig. 2) with the objective of achieving high recoveries (Table 3). In such a case the highest recoveries are in the range 80–90% and, therefore, high enough to detect changes in nucleotide patterns. The analyst must follow the extraction methodology precisely in order to minimize interference from the ion-pairing properties of the impurities [56,57].

3.2. Reversed-phase separation techniques

The reversed-phase separation of purines is performed on C_{18} modified silica columns with a particle size of 3–5 μm. Eluent buffer solutions of

TABLE 3

RECOVERIES OF PURINE COMPOUNDS AFTER EXTRACTION AND NEUTRALIZATION

Extraction with perchloric acid and neutralization with K_2CO_3–triethanolamine. Values are given as percentages of unextracted standards [58].

Purine compound	Recovery (mean ± S.D., n = 6) (%)	
	Albumin-free buffer	Albumin-containing buffer (2%, w/v)
ATP	93 ± 6	84 ± 3
ADP	95 ± 4	78 ± 5
AMP	92 ± 5	75 ± 5
GTP	91 ± 6	81 ± 3
GDP	91 ± 4	82 ± 7
IMP	93 ± 6	87 ± 5
Adenosine	93 ± 5	80 ± 7
Inosine	94 ± 5	85 ± 7
Guanosine	91 ± 7	83 ± 6
Adenine	94 ± 6	79 ± 9
Hypoxanthine	92 ± 5	91 ± 4
Xanthine	95 ± 4	93 ± 3
Uric acid	93 ± 6	90 ± 5
Allopurinol	95 ± 5	86 ± 8
Oxypurinol	90 ± 8	88 ± 4

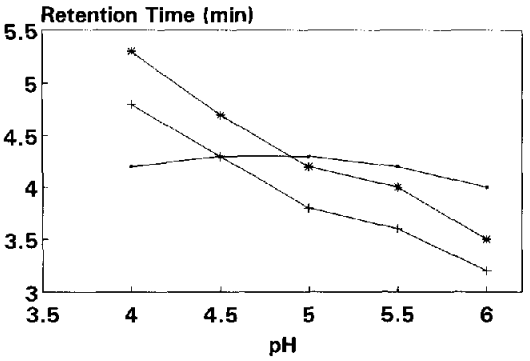

Fig. 3. pH dependence of retention times of (■) adenine, (+) inosine, and (*) guanosine in a reversed-phase system using 10 m*M* ammonium phosphate and 8% methanol as eluent. Chromatographic conditions are described in Fig. 4.

potassium or ammonium phosphate in the concentration range 10–50 m*M* are commonly used [44,59–67]. The hydrophobicity of the eluent is adjusted with methanol or acetonitrile in concentrations between 5 and 50%; the pH range is 4–7. With flow-rates between 1 and 2 ml/min the separation can be performed, in most cases, within 30 min. The mechanism of retention of purine nucleotides and their degradation products has been discussed in depth elsewere particularly by the group of Zakaria *et al.* [59] and Brown *et al.* [65].

3.2.1. Determination of purine nucleosides and bases by reversed-phase separation

Nucleosides and bases are commonly separated by reversed-phase HPLC [59,60]. The determination is generally performed with a low concentration phosphate buffer of *ca.* 10 m*M*, with 5–10% methanol. The pH of the eluent is in the range 4–6 [44]. The elution sequence of nucleosides and bases is pH-dependent. Fig. 3 shows the retention times of guanosine, inosine and adenine. The elution profiles of a standard and an extract of biological sample are shown in Fig. 4. The determination of nucleosides and bases in

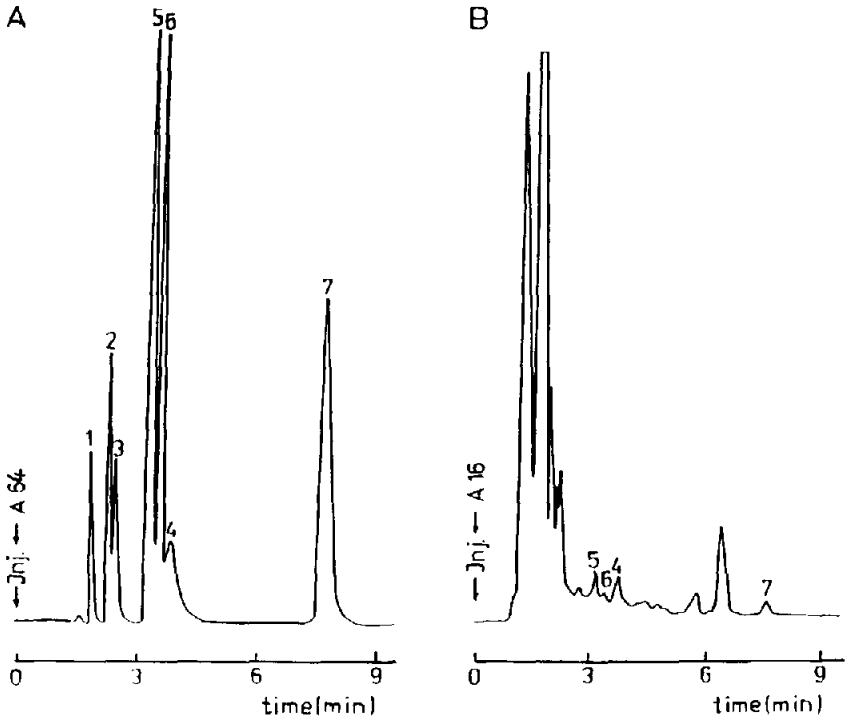

Fig. 4. Analysis of nucleosides and nucleobases in plasma. (A) Standard mixture; (B) plasma. Analysis was performed by a reversed-phase separation technique. Eluent, 10 m*M* potassium phosphate with 8% methanol (pH 5.9); column, 5-μm Nova Pak C_{18} cartridge (100 mm × 8 mm I.D.) with an RCM system. Peaks: 1 = uric acid; 2 = Hyp; 3 = Xan; 4 = Ade; 5 = Ino; 6 = Guo; 7 = Ado. (From ref. 44.)

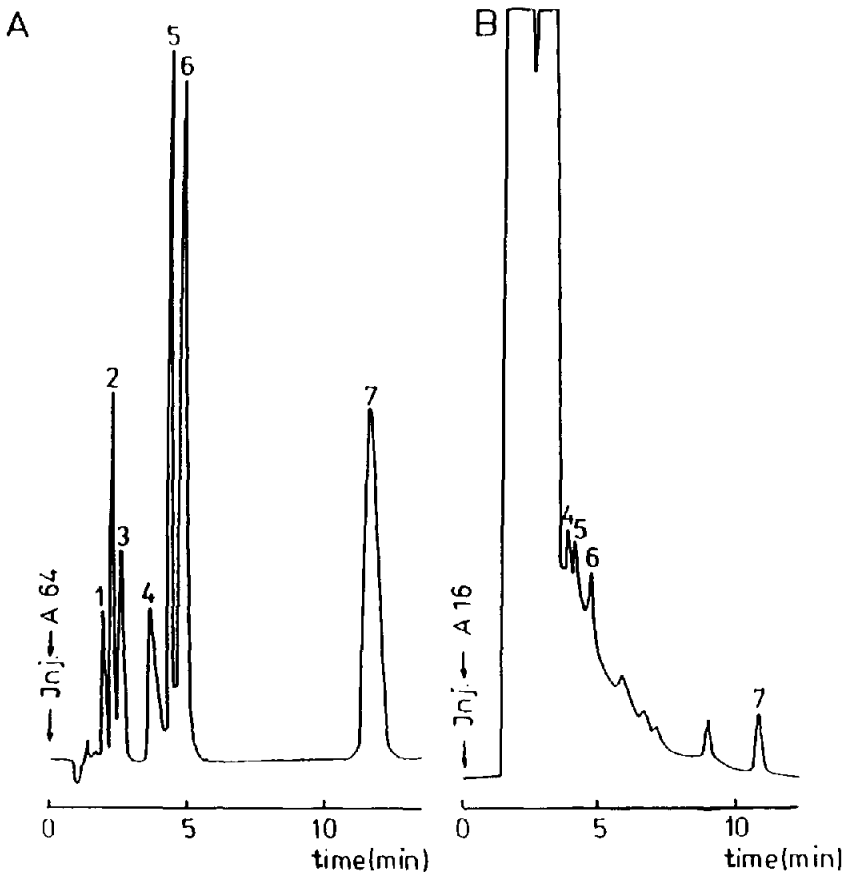

Fig. 5. Nucleoside and nucleobase analysis in cells by isocratic reversed-phase separation. Eluent, 50 m*M* potassium phosphate with 7% methanol (pH 4.1); column, 5-μm Nova Pak C_{18} cartridge (100 mm × 8 mm I.D.) with an RCM system. (A) Standard mixture; (B) skeletal muscle. Peak numbering as in Fig. 4. (From ref. 44.)

cell extracts is shown in Fig. 5. Elution of nucleotides early in the chromatogram in a wide peak can be reduced by a higher concentration of phosphate in the eluent. The more concentrated buffer will reduce the elution interval of the nucleotide peak. In this way it is possible to determine purine nucleosides in cell extracts.

3.2.2. Determination of nucleotides by reversed-phase HPLC

The determination of nucleotides by reversed-phase HPLC has been performed using a concentrated phosphate buffer (0.1–0.15 *M*, pH 6). The organic solvent, methanol, is used to elute only the nucleosides and bases after the analysis of nucleotides, so methanol gradient is increased in most cases after elution of nucleotides [61–67]. Fig. 6 shows the analysis of a nucleotide standard. Because of the concentration differences of nucleosides and the different purine nucleotides (guanylate and adenylate nucleotides) in biological samples (Fig. 7), peak identification at the front of the chromatogram is not easy. Therefore, reversed-phase separation is not often used for the determination of phosphorylated purine compounds, *i.e.* purine nucleotides.

3.3. Ion-pair reversed-phase separation

The determination of purines by ion-pair reversed-phase separation is performed on C_{18} modified silica columns with particle sizes from 3 to 5 μm [66–74], with an eluent of ammonium phosphate buffer (10 m*M*, pH 6–7). Various tet-

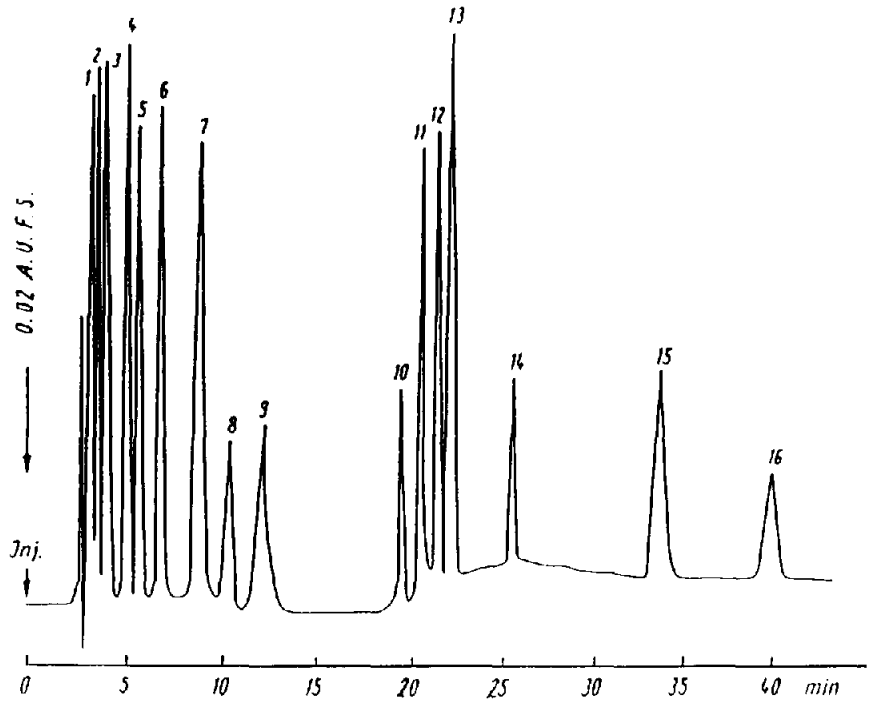

Fig. 6. Nucleotide analysis by reversed-phase HPLC. Analysis was performed with 0.15 *M* potassium dihydrogenphosphate–disodium hydrogenphosphate buffer (pH 6.85), and the same buffer containing 20% methanol (v/v). Column, Zorbax ODS (250 mm × 4.6 mm I.D.). Peaks: 1 = GTP; 2 = GDP; 3 = IMP; 4 = ATP; 5 = ADP; 6 = AMP; 7 = $NADP^+$; 8 = xanthine; 9 = hypoxanthine; 10 = NADPH; 11 = inosine; 12 = guanosine; 13 = NAD^+; 14 = NADH; 15 = adenosine; 16 = adenine. (From ref. 67.)

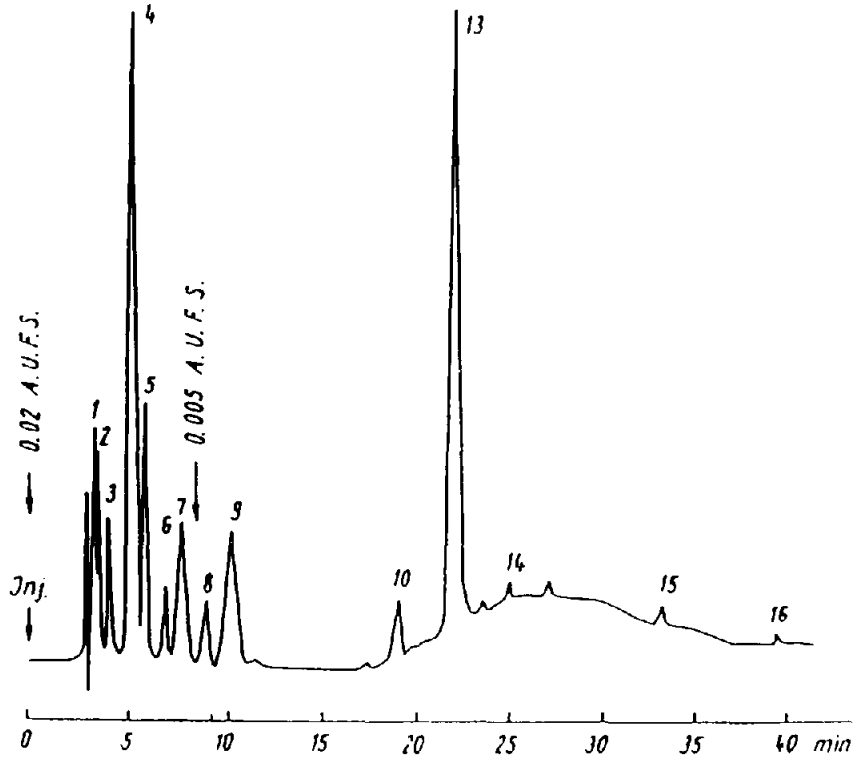

Fig. 7. Separation of a perchloric acid extract of red blood cell suspension. Eluent, column and peaks as in Fig. 6. (From ref. 63.)

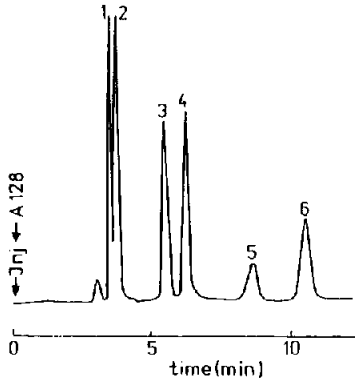

Fig. 8. Analysis of a standard mixture of purine nucleotides by isocratic ion-pair reversed-phase HPLC. Eluent, 10 m*M* ammonium phosphate, 2 m*M* tetrabutylammonium phosphate and 20% acetonitrile; column, 5-μm Nova Pak C_{18} cartridge (100 mm × 8 mm I.D.) with an RCM system. Peaks: 1 = GMP + IMP; 2 = AMP; 3 = GDP; 4 = ADP; 5 = GTP; 6 = ATP. (From ref. 44.)

rabutylammonium salts, usually tetrabutylammonium phosphate, are used as ion-pairing agent, in the concentration range 0.5–10 m*M*. Other ion-pairs are available, *e.g.* Gerret *et al.* [53] and Lim and Peters [75] used TEA. The influence of the pH on the separation quality has been discussed [70,78]. Organic solvents, such as acetonitrile or methanol, are used in concentrations up to 20% in an isocratic or gradient elution mode.

Retention in ion-pair reversed-phase elution can be explained in two ways: (i) formation of an ion-pair between the charged nucleotides and the ion-pair reagent, and (ii) hydrophobic interactions between the ion-pair reagent and the stationary phase, with the nucleotides retained on the "charged" stationary phase [76]. Probably both mechanisms occur in nucleotide retention. The elution times of nucleotides are greater than those of nucleosides and bases, so the sequence of purine elution is the reverse of that in reversed-phase separation techniques.

3.3.1. Determination of nucleotides by isocratic ion-pair reversed-phase HPLC

The elution profile of a typical standard separation of nucleotides is illustrated in Fig. 8. High concentrations of organic solvents, *e.g.* 20% acetonitrile, are used in isocratic elution [44]. Biological samples are frequently separated by this method because of the high sensitivity and stability (high reproducibility) regardless of sample type [69,70]. One of the disadvantages of this method is apparent during the elution of IMP and GMP, two very important purine ribonucleotides. They elute in a single peak (Fig. 9). The difficulties encountered with this method are exacerbated by increased concentrations of perchlorate ions (Fig. 10), which are still present in the samples despite meticulous extraction [56,57] (see also Section 3.1).

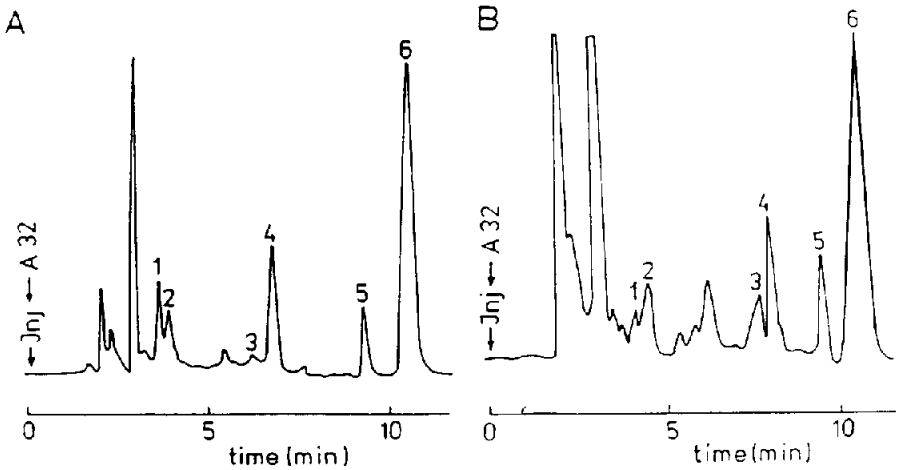

Fig. 9. Nucleotide patterns in erythrocytes (A) and skeletal muscle (B). For chromatographic conditions see Fig. 8. (From ref. 44.)

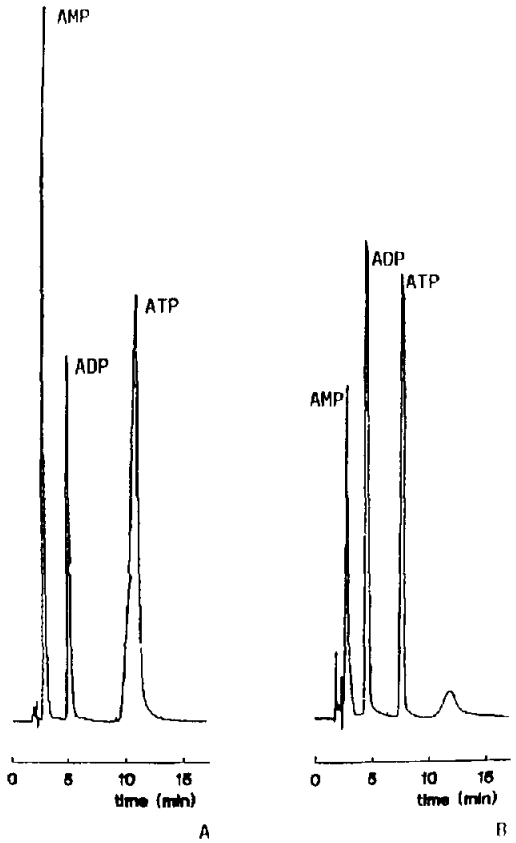

Fig. 10. Effect of perchloric acid on the separation of adenine nucleotide standard mixture. (A) Without perchlorate ions; (B) standard mixture with perchlorate extraction without cooling. Chromatographic conditions as in Fig. 8.

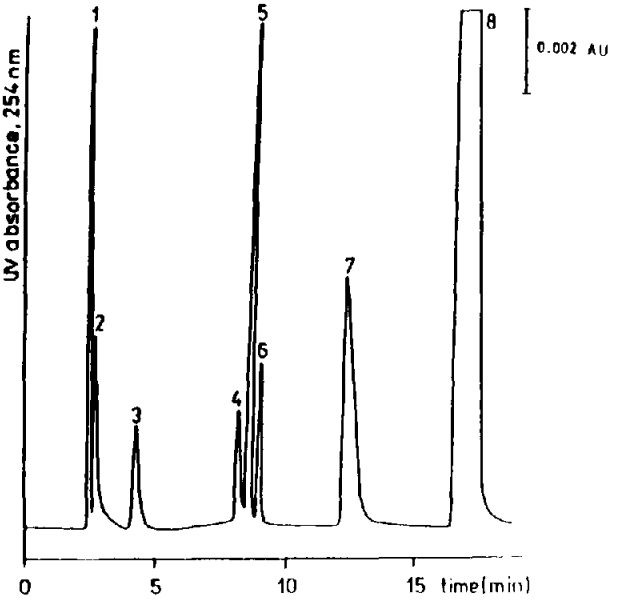

Fig. 11. UV absorbance detection of nucleotide degradation products following isocratic ion-pair reversed-phase HPLC. Mobile phase, 50 m*M* potassium phosphate (pH 5.1), 1 m*M* tetrabutylammonium phosphate and 1% acetonitrile; column, 5-μm Nova Pak C_{18} cartridge (100 mm × 8 mm I.D.) with an RCM system. Peaks: 1 = Hyp; 2 = Xan; 3 = uric acid; 4 = Ade; 5 = Ino; 6 = Guo; 7 = Ado; 8 = AMP. (From ref. 77.)

3.3.2. Determination of monophosphorylated nucleotides, nucleosides and bases by isocratic ion-pair reversed-phase HPLC

For separation of nucleotide degradation products, including the monophosphorylated nucleotides as precursors of nucleotide catabolism (knowledge that is essential in a discussion of the regulation of nucleotide degradation), the eluent consists of a phosphate buffer, the ion-pair reagent and an organic solvent in low concentrations [77]. Fig. 11 shows a typical chromatogram of such a separation. The nucleotides are eluted very late, so that their elution profiles are not detectable. Wide peaks are observed or the compounds are not eluted at all. After a few (*ca.* ten) analyses the columns have to be washed with acetonitrile to remove the retained nucleotides. The nucleobases are eluted early in the chromatogram. Using this separation mode it is possible to detect monophosphorylated nucleotides such as IMP, GMP and AMP, separately. IMP and GMP can be clearly separated using this method and this represents an improvement over previous methods.

3.3.3. Determination of a complex spectrum of purines by gradient ion-pair reversed-phase HPLC

The analysis of a wide range of purine compounds is possible in a gradient of acetonitrile from 0 to 20% in an elution buffer of ammonium phosphate and tetrabutylammonium phosphate (pH 6) [58,71–74]. Separation of a standard mixture is demonstrated in Fig. 12. Elution takes *ca.* 45 min and after this time the column must be equilibrated. A disadvantage of this method is that the elution of the nucleosides while the ace-

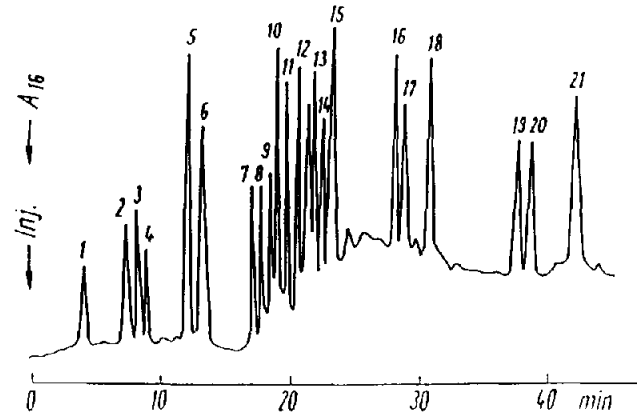

Fig. 12. Chromatogram of a standard mixture of nucleotides, nucleosides and nucleobases. Mobile phase, 10 m*M* ammonium phosphate, 2 m*M* tetrabutylammonium phosphate and acetonitrile (gradient 0–20%); column, C_{18} SIL-X-5 (250 mm × 4.6 mm I.D.). Peaks: 1 = uracil; 2 = hypoxanthine; 3 = uridine; 4 = xanthine; 5 = oxypurinol; 6 = allopurinol; 7 = inosine; 8 = guanosine; 9 = adenine; 10 = adenosine; 11 = NAD^+; 12 = IMP; 13 = GMP; 14 = UMP; 15 = AMP; 16 = GDP; 17 = UDP; 18 = ADP; 19 = GTP; 20 = UTP; 21 = ATP. (From ref. 58.)

tonitrile concentration is increasing makes it difficult to quantify these compounds, owing to the rapidly changing baseline. This point is especially crucial when measuring low levels of nucleosides in tissue.

3.4. Combination of UV and radiochemical detection for flux rate analysis

Radioactivity measurements can be performed with an on-line system, using an HPLC radioactivity detector [77] or by collecting fractions of the eluted compounds [78].

Isocratic elution should be used to measure radioactivity in an on-line system, to obtain identical quenching of the elution solvent during the run. The methods of choice in the determination of nucleotides are isocratic ion-pair reversed-phase separation, and the determination of nucleosides, nucleobases and monophosphorylated nucleotides by a second isocratic ion-pair technique [77]. The lower concentration of acetonitrile (18%) and the lower flow-rate (1.5 ml/min) compared with other separation modes [44] increase the separation time and improve the detection of labelled purines in the large flow-cell of the radioactivity detector. Parallel chromatograms obtained with UV detection and radioactivity measurements are demonstrated in Fig. 13.

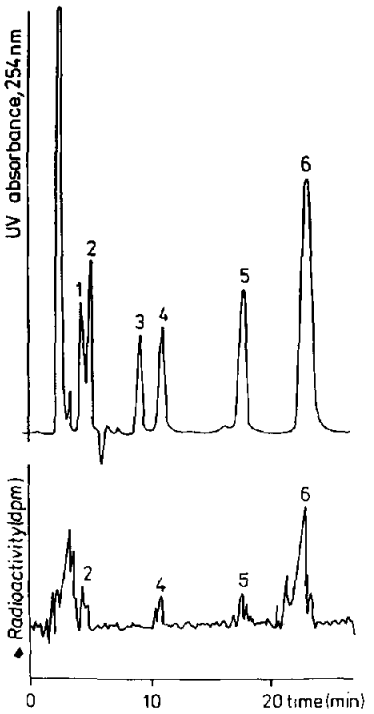

Fig. 13. Chromatograms of separations of nucleotides in an extract of Ehrlich mouse ascites cell suspensions. (Top) Measurement of pool sizes. (Bottom) Detection of radioactivity. Mobile phase, 10 m*M* ammonium phosphate, 2 m*M* tetrabutylammonium phosphate and 18% acetonitrile. Column, 5-μm Nova Pak C_{18} cartridge (100 mm × 8 mm I.D.) with an RCM system. Peaks: 1 = IMP + GMP; 2 = AMP; 3 = GDP; 4 = ADP; 5 = GTP; 6 = ATP. (From ref. 77.)

TABLE 4

NUCLEOTIDE CONTENT IN RED BLOOD CELLS OF DIFFERENT MAMMALIAN SPECIES

Nucleotide	Content (mean ± S.D., n = 5) (mmol/l cells)			
	Rabbit	Mouse	Rat	Human
ATP	1.52 ± 0.05	1.27 ± 0.18	1.48 ± 0.10	1.14 ± 0.07
ADP	0.21 ± 0.03	0.15 ± 0.01	0.22 ± 0.01	0.13 ± 0.01
AMP	0.11 ± 0.02	0.02 ± 0.02	0.11 ± 0.02	0.01 ± 0.003
GTP	0.20 ± 0.04	0.13 ± 0.02	0.20 ± 0.04	0.16 ± 0.01
GDP	0.07 ± 0.01	0.02 ± 0.01	0.07 ± 0.01	0.06 ± 0.008
GMP + IMP	0.05 ± 0.02	0.01 ± 0.005	0.05 ± 0.005	0.07 ± 0.006
ATP/ADP	7.25 ± 0.25	8.47 ± 0.63	6.61 ± 0.44	8.77 ± 0.29
ATP/AMP	13.48 ± 0.46	63.50 ± 2.73	11.27 ± 0.76	114.07 ± 4.28
GTP/GDP	2.74 ± 0.58	6.51 ± 0.27	6.48 ± 1.04	2.67 ± 0.73

4. APPLICATIONS OF REVERSED-PHASE SEPARATION TECHNIQUES TO BIOMEDICAL PROBLEMS

4.1. Purine metabolism in red blood cells

4.1.1. Species comparison

The purine content in the erythrocytes of peripheral blood has been analysed in a great variety of species, *e.g.* mammals [63,67,71,72,79]. Table 4 lists the purine concentrations of red blood cells from different species as measured in our laboratory.

The ATP level in erythrocytes is *ca.* 1.1–1.6 m*M* in cells and is significantly lower than in other organs, such as liver, heart and intestine, with higher metabolic acitivity. The ATP/ADP and ATP/AMP ratios are high in erythrocytes in comparison with other cell types [72]. This is a result of low concentrations of ADP and AMP. The level of GTP is comparable with that of ADP [72,79]. These metabolite levels are not increased, because of the low metabolic activity, low energy consumption and the low turnover of energy-rich nucleotides of erythrocytes.

4.1.2. Comparison of red blood cells of different maturity

Erythrocytes are formed during the erythroporesis in the bone marrow. After differentiation from the red cell precursor, reticulocytes, which are the immediate precursors of mature erythrocytes, are formed. If the life-span of the erythrocytes is shortened, or if they are lost by bleeding, the bone marrow produces more reticulocytes to compensate for the deficiency in oxygen-transporting cells. This is the reason for high yields of reticulocytes in the experimental model of rabbit bleeding anaemia [79]. This biological model can be used to study the content of erythrocytes and reticulocytes. Fig. 14 shows the quasi-linear de-

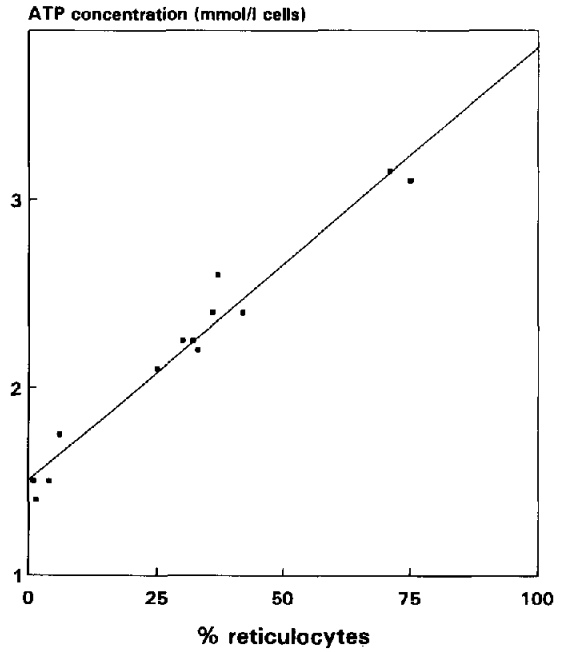

Fig. 14. Relation between ATP concentration and reticulocyte content: y = (0.025 ± 0.00109)x + (1.392 ± 0.0419), r = 0.9813. (From ref. 79.)

TABLE 5

CONCENTRATIONS OF RABBIT PURINE NUCLEOTIDES IN RETICULOCYTE-RICH RED BLOOD CELL SUSPENSIONS AND IN ERYTHROCYTES

Nucleotide	Concentration (mean ± S.D., n = 5) (mmol/l of cells)	
	Erythrocytes	Reticulocytes
ATP	1.52 ± 0.05	2.88 ± 0.22
ADP	0.21 ± 0.03	0.29 ± 0.04
AMP	0.11 ± 0.02	0.13 ± 0.01
GTP	0.20 ± 0.04	0.43 ± 0.04
GDP	0.07 ± 0.01	0.10 + 0.01
GMP + IMP	0.05 ± 0.02	0.07 ± 0.01
ATP/ADP	7.25 ± 0.25	9.65 ± 0.74
ATP/AMP	13.48 ± 0.46	21.31 ± 1.67
GTP/GDP	2.74 ± 0.58	4.51 ± 0.46

pendency of the intracellular ATP concentration on the content of reticulocytes. This dependency was used to extrapolate the levels of purine compounds to 100%. The results of these estimations of purine levels in reticulocytes-rich suspension are demonstrated in Table 5.

The differences of nucleotide levels between mature erythrocytes and reticulocytes are great: between two-fold and three-fold for triphosphorylated purines. Such differences are correlated with loss of cellular organelles (nucleus, mitochondria, intracellular membranes) and different metabolic pathways and cellular functions [80,81] during final maturation to mature erythrocytes. The remaining energy-producing process, glycolysis, is less effective in ATP synthesis than the mitochondrial respiratory chain.

Erythrocytes undergo various changes and damage during their lifetime in the bloodstream. These changes are due to effects of reactive oxygen species, and to variations of osmolarity in different parts of the bloodstream, such as the kidney, etc. Loss of water with increasing age causes the cell structure to deform. Erythrocytes are not able to compensate such changes because of the lack of a protein synthesis mechanism. One result of such changes is evidenced by the existence of different density fractions of erythrocytes [82], which can be separated by density gradient centrifugation. The concentrations of adenine nucleotides in various density gradient fractions (Fig. 15) show that the adenine nucleotide content parallels changes that occur during ageing [83].

4.1.3. Purine nucleotide content of red blood cells of uremic patients

One possible disturbance of erythrocyte formation in the bone marrow is a deficiency of

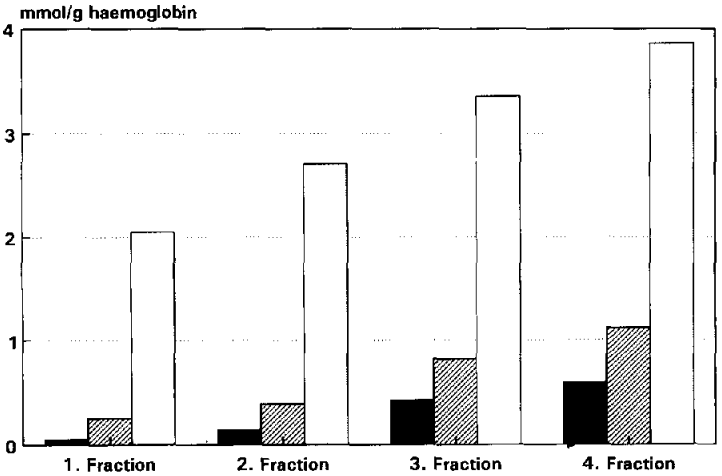

Fig. 15. Concentration of adenine nucleotides in different density fractions of human erythrocytes (dotted bar, ATP; striped bar, ADP; black bar, AMP).

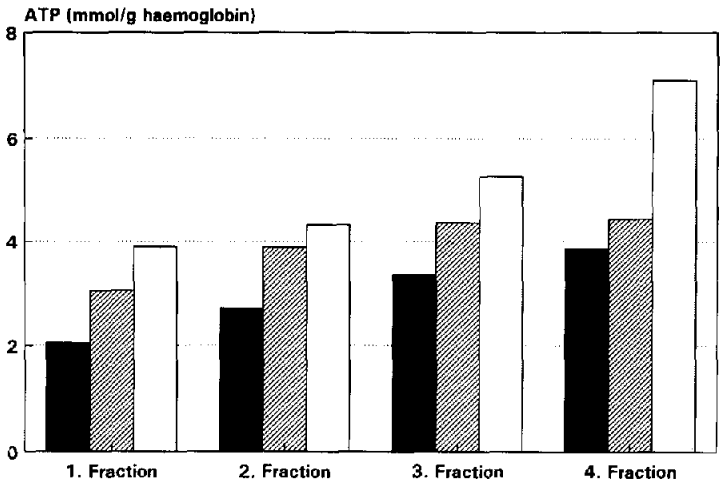

Fig. 16. ATP content in different density fractions of human erythrocytes (black bar, without disease; striped bar, chronic renal insufficiency; dotted bar, chronic renal insufficiency with erythropoietin treatment).

erythropoietin. Erythropoietin is formed mainly in the kidney, so patients with chronic renal failure do not produce this peptide [84]. This deficiency leads to a drastic reduction in red blood cell formation. Furthermore, uremic changes in blood plasma lead to a decreased lifetime of circulating erythrocytes. Both these effects lead to serious anaemia in patients with chronic renal failure. The metabolism of erythrocytes in these patients is greatly changed [85], including purine nucleotide metabolism. Treating these patients with recombinant human erythropoietin influences metabolic conditions during the maturation cycle of red blood cells. This was the evidenced by an increase in nucleotide levels, as demonstrated for ATP in Fig. 16.

4.1.4. Changes in purine pattern during oxidative damage of red blood cells

The investigation of changes in cellular energy and nucleotide metabolism during conditions of oxidative stress is of great medical significance. Various models of erythrocytic damage during oxidative stress have been used, *e.g.* hydrogen peroxide [86], superoxide radical and hydrogen peroxide formation by xanthine oxidase and xanthine [87], phenylhydrazine addition [29], etc. Results of nucleotide degradation in erythrocytes after phenylhydrazine treatment are shown in Table 6. The degradation of the nucleotides is connected with (i) a direct reaction of the phenylhydrazine products with the nucleotides, (ii) an initiation of free radical chain reactions under involvement of oxygen and (iii) an energy imbalance in the cells after membrane damage [88].

4.2. Purines in the gastrointestinal tract

4.2.1. Comparison of different cells and organs

A great number of cell types with different functions are located in the gastrointestinal tract. Some of these cells, such as hepatocytes or enterocytes, the parenchymal cells of the liver and the intestine, perform the primary function of the gastrointestinal tract. These cells are in tight contact with a complex of other cells, such as fat cells, muscle or endothelium cells. Table 7 lists the purine content of some organs and isolated cell types of the gastrointestinal tract. The nucleotide content in metabolically active cells, such as hepatocytes or enterocytes, is high in comparison with that of red blood cells [58]. The concentration of guanine nucleotides in hepatocytes is higher than in other cell types, because of their involvement in many liver-specific metabolic pathways. The relatively high amounts of di- and

TABLE 6

CONCENTRATIONS OF RABBIT PURINE NUCLEOTIDES IN ERYTHROCYTE SUSPENSIONS AFTER INCUBATION WITH AND WITHOUT THE ADDITION OF PHENYLHYDRAZINE

Incubation time, 90 min.

Nucleotide	Concentration (mean ± S.D., n = 5) (mmol/l of cells)	
	Without addition	With addition of 20 mM phenylhydrazine
ATP	1.52 ± 0.05	0.46 ± 0.08
ADP	0.21 ± 0.03	0.27 ± 0.08
AMP	0.11 ± 0.02	0.13 ± 0.01
GTP	0.20 ± 0.04	0.16 ± 0.06
GDP	0.07 ± 0.01	0.12 ± 0.02
GMP + IMP	0.05 ± 0.02	0.05 ± 0.01
ATP/ADP	7.25 ± 0.25	1.73 ± 0.30
ATP/AMP	13.48 ± 0.46	3.46 ± 0.60
GTP/GDP	2.74 ± 0.58	1.29 ± 0.47

monophosphorylated compounds in comparison with the corresponding triphosphorylated nucleotides are due to the high energy turnover in these cells [89].

Isolated cells often have been used for the investigation of metabolic pathways and their changes under pathological conditions. Most of these experiments were carried out with hepatocytes. There are reports on studies with other cell types, including enterocytes. The dissociation and isolation are facilitated by using enzymes, such as collagenase, trypsin or elastase [90], by calcium depletion [91] or by cold shock [92], and, therefore, involve damage and stress to the cells. That could be the reason for slight decreases of nucleotide concentrations in isolated cells compared with values determined by assays using the intact organ (see Fig. 17).

TABLE 7

NUCLEOTIDE CONCENTRATIONS IN DIFFERENT ORGANS OF THE GASTROINTESTINAL TRACT

Data from refs. 44, 58, 94 and 96.

Nucleotide	Concentration (mmol/l of cells)				
	Rat liver[a]	Rat hepatocytes	Rat small intestine	Rat colon	Mouse liver
ATP	4.05 ± 0.47	2.16 ± 0.17	2.55 ± 0.57	1.95 ± 0.32	1.51 ± 0.17
ADP	1.42 ± 0.26	0.43 ± 0.04	0.87 ± 0.18	0.22 ± 0.09	1.06 ± 0.04
AMP	0.52 ± 0.12	0.27 ± 0.03	0.73 ± 0.25	0.09 ± 0.02	0.27 ± 0.01
GTP	1.73 ± 0.09	0.33 ± 0.02	1.24 ± 0.09	0.38 ± 0.05	0.50 ± 0.15
GDP	0.41 ± 0.19	0.06 ± 0.03	0.73 ± 0.18	0.09 ± 0.04	0.09 ± 0.02

[a] Unpublished data.

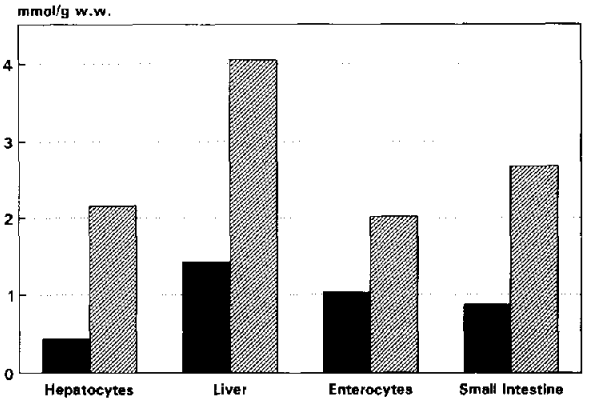

Fig. 17. Content of ATP (striped bar) and ADP (black bar) in isolated hepatocytes, isolated enterocytes, liver and small intestine.

4.2.2. Hypoxia and reoxigenation in the liver

The degradation of ATP and GTP during oxygen deficiency in isolated hepatocytes has been well investigated [58]. The ATP concentration in isolated hepatocytes decreases to *ca.* 50% within 7 min of anoxia and approaches *ca.* 10% after 30 min, as shown in Fig. 18. Drastic depletion of ATP is followed by an increase in purine catabolites: peak concentrations of these occur at 10 min (ADP), 20 min (AMP) and 30 min (IMP and adenosine). Fig. 19 illustrates the wave-like behaviour of ATP catabolites [93]. In the presence of oxypurinol, an inhibitor of xanthine oxidoreductase, the hypoxanthine concentration at normox-

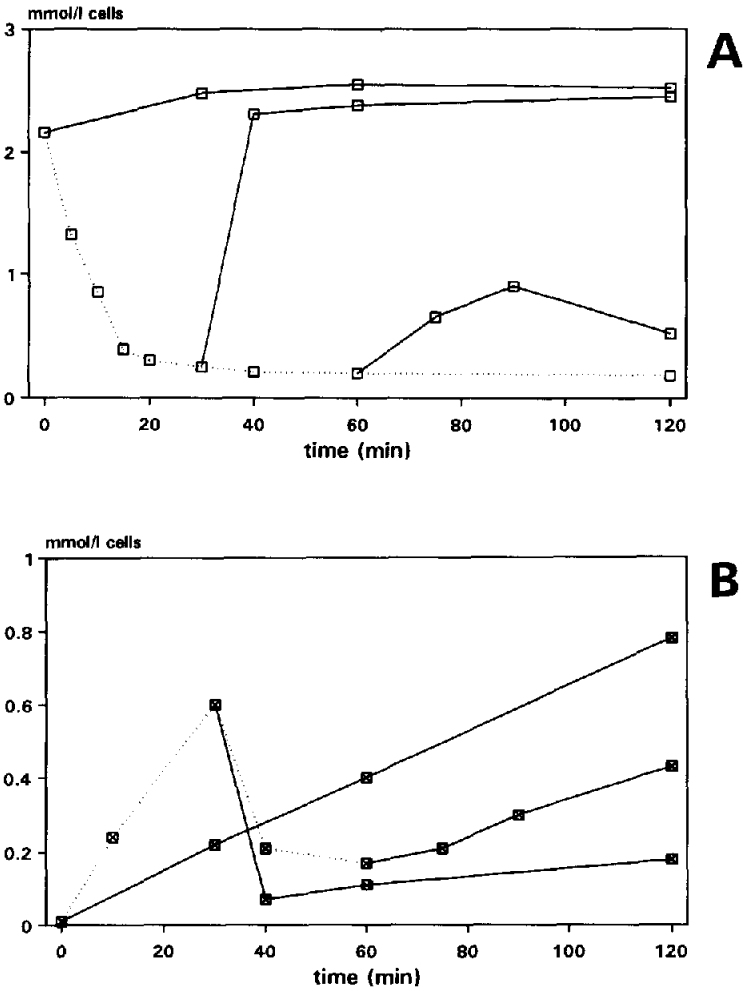

Fig. 18. (A) ATP and (B) hypoxanthine concentrations in isolated hepatocytes during ischemia (0, 30, 60 min) (···) and normoxia or postanoxic reoxygenation (———).

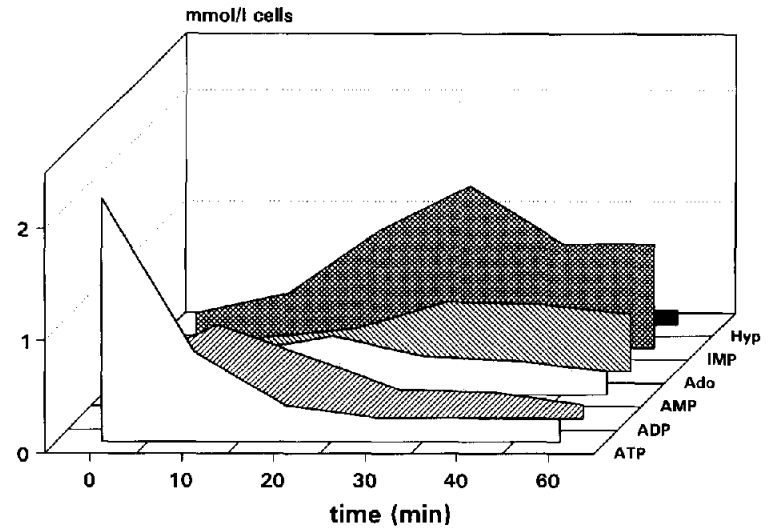

Fig. 19. Changes of ATP, ADP, AMP, adenosine, IMP and hypoxanthine concentrations of rat hepatocytes during anoxia experiments.

ia increased proportionally with time [93]. During anoxia the increase is more drastic. Fig. 18 shows an increase in hypoxanthine concentration under anoxic conditions. The presence of oxypurinol prevents the further metabolism of hypoxanthine to uric acid and allantoin. One may therefore conclude that the difference in hypoxanthine level in the presence or absence of oxypurinol reflects the metabolic activity of xanthine oxidoreductase, *i.e.* the flux from hypoxanthine to xanthine (first reaction of the xanthine oxidoreductase). The flux rate via the second reaction step catalysed by xanthine oxidoreductase will be somewhat greater, because of the additional influx into the xanthine pool from guanine catalysed by guanase. Our hepatocyte experiments show no distinct increase of uric acid concentration in the absence of xanthine oxidoreductase inhibitors. This suggests that uric acid generated by uricase rapidly degrades and is not detectable by either UV detection at 254 nm or other spectrophotometric methods. Uricase, which is not present in the liver and other organs of primates, has a high activity in rat liver.

4.2.3. Ischemia and reperfusion of the small intestine

The degradation of triphosphorylated nucleotides in the small intestine is similar to that observed in hepatocytes (Table 8; data from refs. 94 and 95). However, it is less rapid in intact organs than in isolated cells. Cell–cell interactions are not interrupted, and the oxygen stress can be compensated for a longer time in the intact organ (*in vivo* or under isolated organ perfusion conditions). After 1 h of ischemia, only half the ATP was degraded in the small intestine of rats. The equivalent value for oxygen deficiency in rat hepatocytes was 90% of the ATP degraded (see above). Also, the processes of restoration of nucleotide pools are slower in organs such as the small intestine (Table 8, Fig. 18). The degradation of ATP in the small intestine is also accompanied by a temporary increase of the levels of purine degradation products and hypoxanthine [94]. In the small intestine, a marked acceleration of ATP recovery in the presence of oxypurinol was measured.

4.2.4. Intestinal inflammation

Chronic inflammatory intestinal diseases, such as Morbus Crohn and colitis ulcerosa, are debilitating and often lead to inability to work, and often require radical surgical procedures to correct. The need for an increasing understanding of the aetiology of such diseases, and the search for

TABLE 8

CONCENTRATIONS OF PURINES DURING ANOXIA AND REPERFUSION OF RAT SMALL INTESTINE

Data from refs. 94 and 95.

Compound	Concentration (nmol/mg of protein)			
	0 min	60 min ischemia	10 min reperfusion	20 min reperfusion
Uric acid	1.74 ± 0.42	1.90 ± 0.42	2.19 ± 0.27	2.99 ± 0.50
Hyp	0.04 ± 0.01	0.81 ± 0.27	0.52 ± 0.15	0.42 ± 0.07
IMP + GMP	1.3 ± 0.3	3.2 ± 0.9	1.7 ± 0.5	1.2 ± 0.3
GDP	3.2 ± 0.8	1.8 ± 0.6	2.8 ± 0.7	2.4 ± 0.6
GTP	5.4 ± 0.4	3.7 ± 0.8	3.8 ± 0.7	3.4 ± 0.9
AMP	3.2 ± 1.1	3.2 ± 0.8	3.0 ± 1.1	2.2 ± 0.7
ADP	3.8 ± 0.8	4.5 ± 0.6	5.3 ± 1.0	4.5 ± 0.4
ATP	11.1 ± 2.5	4.5 ± 1.1	4.6 ± 1.4	5.8 ± 1.7

more effective and novel treatments, led to the development of experimental animal models, such as the one using trinitrobenzenesulphonic acid instillation. In this model, a single instillation of the trinitrobenzenesulphonic acid into the rat colon produces chronic ulceration and inflammation [96]. After treatment with this compound the ATP content of the tissue and the total concentration of adenine nucleotides and the GTP concentration are markedly decreased (Table 9; data from ref. 96). Oxypurinol treatment prevented the decline of ATP and GTP concentrations after trinitrobenzenesulphonic acid treatment [96,97]. This protective action can be explained by (i) an acceleration of salvage pathways of purine metabolism, (ii) a decrease of radical formation mediated by xanthine oxidase because of the inhibition of this enzyme by oxypurinol and (iii) the radical scavenging effect of oxypurinol.

TABLE 9

NUCLEOTIDE CONCENTRATIONS AND ATP/ADP, ATP/AMP AND GTP/GDP RATIOS IN THE COLON OF RATS FOLLOWING THE RECTAL INSTILLATION OF 0.25 ml OF 30% ETHANOL WITH 25 mg OF TRINITROBENZENESULPHONIC ACID

Data from ref. 96.

Nucleotide	Concentration (μmol/g wet weight)		
	Control	TNB	TNB + oxypurinol
ATP	1.95	1.10	1.81
ADP	0.22	0.29	0.30
AMP	0.09	0.19	0.10
GTP	0.38	0.14	0.35
GDP	0.09	0.12	0.08
ATP/ADP	8.86	3.79	6.03
ATP/AMP	21.67	5.79	18.10
GTP/GDP	4.22	1.17	4.38

4.3. Other organs and tissues

The nucleotide concentrations of various other organs are listed in the Table 10. The nucleotide content of the kidney is comparable with that of the liver because of the high metabolic activity of this organ (*i.e.* the increased energy demand for transport processes in proximal and distal tubuli). It is difficult to estimate the concentrations of purines in the kidney cortex. Preparation of the cortex by breaking the organ after freezing it in liquid nitrogen is complicated. This may be the reason for the unexpectedly low ATP/ADP ratio that we measured (Table 10).

In skeletal muscle tissue, ATP metabolism is closely connected with the creatine phosphate pool. Adenine nucleotide metabolism is acceler-

TABLE 10

PURINE NUCLEOTIDE CONCENTRATIONS IN KIDNEY OF RATS AND SKELETAL MUSCLE OF MICE

Data from ref. 44.

Nucleotide	Concentration (μmol/g wet weight)		
	Kidney[a] (whole organ)	Kidney cortex[a]	Skeletal muscle
ATP	2.56 ± 0.23	1.21 ± 0.37	2.40 ± 0.22
ADP	1.66 ± 0.52	1.89 ± 0.63	0.37 ± 0.02
AMP	0.82 ± 0.15	0.91 ± 0.29	0.06 ± 0.02
GTP	1.51 ± 0.26	0.93 ± 0.51	0.14 ± 0.01
GDP	0.59 ± 0.32	0.65 ± 0.29	0.03 ± 0.01

[a] Unpublished data.

ated owing to the involvement of nucleotides in the contraction processes. That rapid energy turnover, including the high ATP delivery, requires high ATP/ADP and ATP/AMP ratios in muscle cells.

4.4. Purine components in body fluids

Blood plasma [13,44,98–100], cerebrospinal fluid [99,101] and synovial fluid [31] have been investigated. Under various conditions both the chemical composition and the volume of some body fluids can increase. The most prominent of such volume changes is the formation of amniotic fluid [99] during pregnancy and the formation of ascites fluid [44] during pathogenesis of different tumours and cirrhotic liver diseases. In all these (extracellular) fluids the concentration of nucleotides is very low and is often, for several metabolites that are interesting for our investigations, lower than the detection limit. Nucleosides and bases can rapidly pass through the cell membrane, leading to approximately equal concentrations of nucleosides and bases outside and inside the cells.

We studied purine concentrations in blood plasma (human, mouse), cerebrospinal fluid (human) and ascites fluid (mouse), using reversed-phase HPLC. The concentrations of some of purine compounds which were measured in these body fluids are shown in Table 11. The purines in plasma reflect the interrelated nature of the pu-

TABLE 11

CONCENTRATION OF PURINE DEGRADATION PRODUCTS IN BODY FLUIDS

Data from ref. 44.

Nucleotide	Concentration (μM)		
	Mouse plasma	Ascites fluid (Ehrlich mouse ascites tumour)	Newborn human cerebrospinal fluid [a]
Ado	1.2 ± 0.3	0.03 ± 0.01	N.D.
Ade	3.2 ± 0.9	1.3 ± 0.2	8.23 ± 1.99
Ino	2.1 ± 0.4	1.3 ± 0.1	3.93 + 0.71
Guo	0.8 ± 0.1	0.5 ± 0.1	N.D.

[a] Unpublished data.

rine metabolism of different organs, *i.e.* the interorgan relationships. With these interrelationships the interorgan exchange of purines between organs with high *de novo* synthesis, *e.g.* liver, and organs with purine demand but low activities of enzymes for *de novo* synthesis, such as the bone marrow with its fast proliferation processes, seems to be of major importance.

4.5. Purine metabolism of Ehrlich ascites tumour cells: a classical and representative model of tumour proliferation

4.5.1. Purine content in tumour cells and host tissues in different proliferation phases of the tumour

During the *in vivo* growth of Ehrlich ascites tumours there is a proliferating phase, in which the number of cells increase quasi-exponentially, followed by a resting phase, in which the number of cells stays practically constant [102]. The growth phases are characterized by structural deterioration and a decreased number of mitochondria, decreased substrate utilization, changes in protein synthesis and decreased uptake of radiolabelled purine precursors, such as adenine, hypoxanthine and inosine [18,103,104]. Decreased energy production in the resting phase is accompanied by changes in purine pattern [67,77,105], as demonstrated in Table 12. The changes in nucleotide pattern are connected with different changes of nucleotide concentrations in the host organs in tumour-bearing animals (Table 13: data from refs. 44 and 105).

TABLE 12

NUCLEOTIDE CONCENTRATIONS AND RATIOS BETWEEN NUCLEOTIDE CONCENTRATIONS IN EHRLICH MOUSE ASCITES TUMOUR CELLS

Data from ref. 105.

Nucleotide	Concentration (nmol/g wet weight)	
	Proliferating phase	Resting phase
ATP	3047 ± 176	1136 ± 151
ADP	629 ± 60	310 ± 37
AMP	227 ± 22	150 ± 25
GTP	799 ± 75	291 ± 47
GDP	244 ± 12	70 ± 9
ATP/ADP	4.84 ± 0.28	3.66 ± 0.49
ATP/AMP	13.42 ± 0.78	7.57 ± 1.01
GTP/GDP	3.27 ± 0.31	4.16 ± 0.17

TABLE 13

CONCENTRATIONS OF ADENINE NUCLEOTIDES IN HOST TISSUES OF HEALTHY CONTROL ANIMALS AND OF EHRLICH ASCITES TUMOUR-BEARING ANIMALS DURING THE PROLIFERATING AND RESTING PHASES OF TUMOUR GROWTH

Data from ref. 44.

	Concentration (nmol/g wet weight)		
	Liver	Skeletal muscle	Erythrocytes
ATP			
Control	1512 ± 172	2401 ± 224	1702 ± 443
Proliferating	1904 ± 122	2839 ± 367	2017 ± 163
Resting	1546 ± 185	1508 ± 308	2020 ± 110
ADP			
Control	1059 ± 38	375 ± 23	174 ± 25
Proliferating	1301 ± 190	467 ± 53	198 ± 54
Resting	1052 ± 123	519 ± 151	513 ± 106
AMP			
Control	269 ± 13	61 ± 19	37 ± 17
Proliferating	319 ± 50	54 ± 11	78 ± 18
Resting	262 ± 48	116 ± 12	242 ± 81

4.5.2. Changes of purine pattern during reactivation of resting tumour cells

The transition of tumour cells from the stationary resting phase to the growth phase is of great medical importance, *e.g.* during metastatic processes and during reactivation of quiescent tumour cells that were not destroyed by radiological or chemical treatment. The process of reactivation (or rejuvenation) of those tumour cells could be modelled in an *in vitro* culture of Ehrlich ascites tumour cells, which were harvested from tumour-bearing mice during the resting phase of tumour growth. In a complete cultivation medium, containing high concentrations of substrates and cosubstrates, the concentration of ATP in these cells increases rapidly to a level that is higher than that observed during the proliferating phase of growth. In the later phases of such experiments (*ca.* 4 h) the ATP concentration of ascites cells approaches the ATP level of the cells in the proliferating phase of tumour growth (Fig. 20; data from ref. 106).

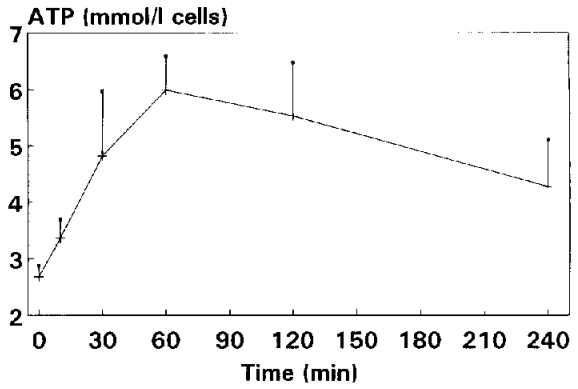

Fig. 20. ATP concentrations of Ehrlich ascites tumour cells of resting phase in an *in vitro* culture (mean ± S.D.).

4.5.3. Purine metabolism in the different growth phases of the tumour

Table 14 shows the concentration of the specific activities and total activities of the purine pools after 30 min of incubation of the Ehrlich ascites tumour cells in the presence of ^{14}C-labelled adenine as radioactive precursor. The distribution of the radioactivity in the different purine compounds is higher in most compounds in the cells in the proliferating phase of tumour growth, suggesting higher enzyme activities and flux-rates in nucleotide metabolism in these cells compared with cells in the plateau phase [77,107]. The dynamics of the radioactivity in single purine compounds is demonstrated in a series of chromatograms in Fig. 21. The increased amount of radioactivity in the ATP pool is the result of a high influx rate in this pool, leading to an increase in specific radioactivity (dpm/nmol).

TABLE 14

CONCENTRATIONS, SPECIFIC RADIOACTIVITIES AND POOL RADIOACTIVITIES OF PURINE COMPOUNDS OF EHRLICH ASCITES TUMOUR CELLS OF THE PROLIFERATING GROWTH PHASE

Data obtained after 30 min incubation in presence of [^{14}C]adenine at 2% cytocrite [77].

Nucleotide	Concentration (μmol/l suspension)	Specific radioactivity (dpm/nmol)	Pool radioactivity (dpm)
ATP	92.2	1545	141 477
ADP	31.1	915	28 373
AMP	13.3	2365	30 746
GTP	18.7	995	63 162
IMP + GMP	6.2	2325	14 407
Ado	0.45	45 509	20 479
Ade	0.98	485 985	476 265
Hyp	1.55	18 946	29 366
Uric acid	3.37	6289	21 197

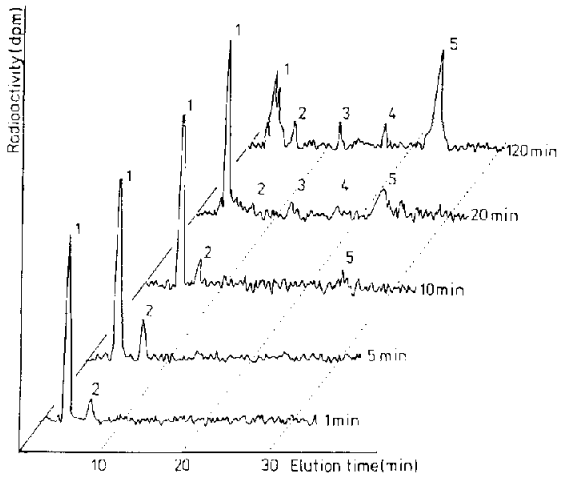

Fig. 21. Changes of nucleotide chromatograms (radioactive detection) during incubation of Ehrlich mouse ascites cells with [^{14}C]adenine. (From ref. 107.)

5. ADVANTAGES AND DISADVANTAGES OF THE DIFFERENT SEPARATION TECHNIQUES

This review has documented a number of methods, and the application of these methods to biological and medical problems and to different tissues and organs. The choice of method for the determination of low-molecular-mass purine compounds depends on the biomedical problems that are investigated, which group of metabolites has to be analysed, which tissue, organ or body fluid is being sampled and which impurities can influence the determinations. The different separation methods, with their corresponding advantages and disadvantages, allow the determination of purine ribonucleotides in a great number of tissues. They also allow the interpretation of metabolic changes in the course of various physiological changes, such as tumour development, chronic renal failure, hypoxia and ischemia in gastrointestinal organs, inflammatory bowel disease, oxidative stress and toxicological cell destruction. Table 15 summarizes the studies in our laboratory using HPLC separation of purine nucleotides, nucleosides and bases. The table includes brief notes of the advantages and disadvantages of each separation method.

TABLE 15

REVERSED-PHASE TECHNIQUES FOR SEPARATION OF PURINE NUCLEOTIDES, NUCLEOSIDES AND BASES

Metabolite(s)	Advantages/disadvantages	Reference(s)
Isocratic reversed-phase HPLC		
Nucleotides	High buffer concentrations Low separation quality	61,67
Nucleosides and bases	High reproducibility In cell extracts only nucleosides Short separation times	44
Gradient reversed-phase HPLC		
Nucleotides, nucleosides and bases	High salt concentrations Low separation quality	61,67
Isocratic ion-pair reversed-phase HPLC		
Nucleotides	High reproducibility Short separation times Influences by perchlorate ions Usable for radiochemical detection	44,67,77
Nucleosides, bases and monophosphorylated nucleotides	Short separation times Wide metabolite spectra Usable for radiochemical detection	77
Gradient ion-pair reversed-phase HPLC		
Nucleotides, nucleosides and bases	Wide metabolite spectra Long separation times Low reproducibility Influenced by extraction modes	68

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